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PENETRATION ENZYMES OF SCHISTOSOME CERCARIAE.(U)
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BACKGROUND

It is the purpose of this program to study in depth the invasive process by which schistosome cercariae infect their vertebrate hosts. Skin invasion involves muscular activity and secretion from the post- and preacetabular glands of cercariae. Preacetabular secretion contains proteolytic enzyme(s) which is secreted deep into skin tissue. This enzyme(s) is considered to modify some component of these tissues, facilitating the larval schistosomes' migration from skin horny layer into venules. Clarification of the relationships between this secreted enzyme(s) and the level of cercarial production by snail hosts on one hand and cercarial infectivity for vertebrate hosts on the other hand has been one focus of attention. Infectivity of cercariae was measured by their ability first to penetrate skin, and second to mature in mice. Other areas of investigation were as follows. A second focus of attention was on the finding that concentrated secretion appeared to have an inhibitory effect on the secretion of fresh emzyme. A third concern (with Dr. Murrell, NMRI) was cryopreservation of irradiated schistosomules to be used in preparation of a schistosome vaccine; a fourth, (with Dr. Dorsey, NMRI) structure and function of the cercarial penetration organs; a fifth (with Drs. Minard and Murrell, NMRI) exploration of the immunogenicity of collected preacetabular gland secretion; and a sixth (with Drs. Dorsey, NMRI, and Cousin, NIH postdoctoral

Fellow at BRI), evaluation and critical comparison of early postpenetration larvae (schistosomules) produced by different methods.

OBJECTIVE I. TO ESTABLISH BASE LINES OF DAY-TO-DAY VARIABILITY OF SECRETED ENZYME AND TO RECOGNIZE ANY CORRELATION BETWEEN LEVELS OF ENZYME ACTIVITY AND CERCARIAL PRODUCTION AND INFECTIVITY

METHODOLOGY

A. Snails were individually exposed, as in our routine protocol, to 8 to 10 miracidia from a single miracidial pool and maintained until their infections were patent as tested by cercarial emergence. This required about 5 weeks.

Cercariae were collected from this group of snails (an isolated group to which no other snails were added), and pooled each Tuesday and Friday after the first day on which enough emerged for study. Cercarial numbers were calculated each collection day from counts of 6 aliquots of 0.5 ml of cercarial suspension and the average number per snail was recorded.

Enzyme was harvested over linolenic acid from 7000 cercariae/ml in 10 ml volumes and its activity tested in vitro against the dye-coupled collagen, Azocoll, by the techniques developed during FY 1976 and described in Annual Report No. 1.

To test infectivity, three mice were exposed by tail to 50 cercariae from the common pool for the day. The number

penetrating was calculated for each mouse by subtracting from the original 50 cercariae, the number which did not penetrate. Numbers of parasites maturing in each mouse were established 7 weeks later by manual recovery of the adult worms.

Numbers of snail deaths were recorded on each collection day. Results were recorded as long as sufficient numbers of cercariae could be collected. Data were graphed for each parameter and are shown in Figure 1.

A replication (incomplete, in that worm burdens are not yet available) (Fig. 1) varied in design from the above in that 50 cercariae/ml were used for enzyme collection instead of 7000. This modification was necessary because a new shipment of Azocoll had to be used. Each bottle of Azocoll substrate must be calibrated and the required adjustment made to remain within linearity of the spectrophotometer.

B. In an attempt to reduce the variability inherent in infections of 8 to 10 miracidia per snail by synchronizing the parasites' development, a similar experiment was designed using cercariae from a group of snails exposed individually to 1 miracidium. All miracidia were from a common pool. Six weeks postexposure, 58 of the 100 snails exposed had patent infections. Of these, 5 were isolated to provide cercariae each Tuesday and Friday. The same procedures were used and parameters measured as for the original and replication using multiply infected snails. Data are given in Figure 2.

RESULTS

A. Cercariae from multiply-infected snails (8 to 10 miracidia per snail). Results are available from the first experiment and one replication. The following variables were tested (Fig. 1): proteolytic enzyme activity; snail deaths; average cercarial emergence per snail; % cercariae penetrating; and % worms maturing.

One attempted replication failed because of the early death from some unknown cause of many of the infected snails. Another replication is complete except for matured worm burdens (Fig. 1).

Day-to-day variability was characteristic of all parameters. Inspection of the curve of enzyme activity in the first experiment, however, suggested a pattern (Fig. 1). There was an initial period of relatively low activity, a mid-period of higher, and a final period again of low activity, as measured by digestion of Azocoll read spectrophotometrically as absorbance at 520 nm. Averages and ranges of absorbance readings, average cercarial production and percentages of cercarial penetration and worm maturation for the 3 periods are tabulated below.

PERIOD	POST-EXPOSURE DAYS	AV. ENZYME ACTIVITY ABSORBANCE	AV. NO. CERC/SNAIL ($X10^3$)	AV. % CERCARIAL PENETRATION	AV. % WORM MATURATION
Initial low	34-65	0.325	2.7	90	42
Mid high	69-111	0.421	3.8	94	40
Final low	114-128	0.358	2.4	90	42

Legend for Figure 1. Activity of enzyme secreted by cercariae compared with cercarial production by the same snails and cercarial infectivity for mice. Data are from two isolated snail groups which varied in composition only by snail deaths. Snails were exposed to 8 to 10 miracidia each. Data from the first group of snails are shown by broken lines; from the second group, by solid lines. Figures below the abscissa indicate the snail postexposure day on which the cercariae were collected: numerators, the broken lines and denominators, the solid lines. For enzyme activity, given in absorbance at 520 nm, x's in the broken line represent the average of 3 tubes each containing 1 ml of Azocoll substrate and 0.5 ml of secretion collected from 3500 cercariae and dots show the minimal and maximal figures. Symbols are the same for the solid line, but here each point indicates activity of 1 ml of enzyme secreted by 50 cercariae.

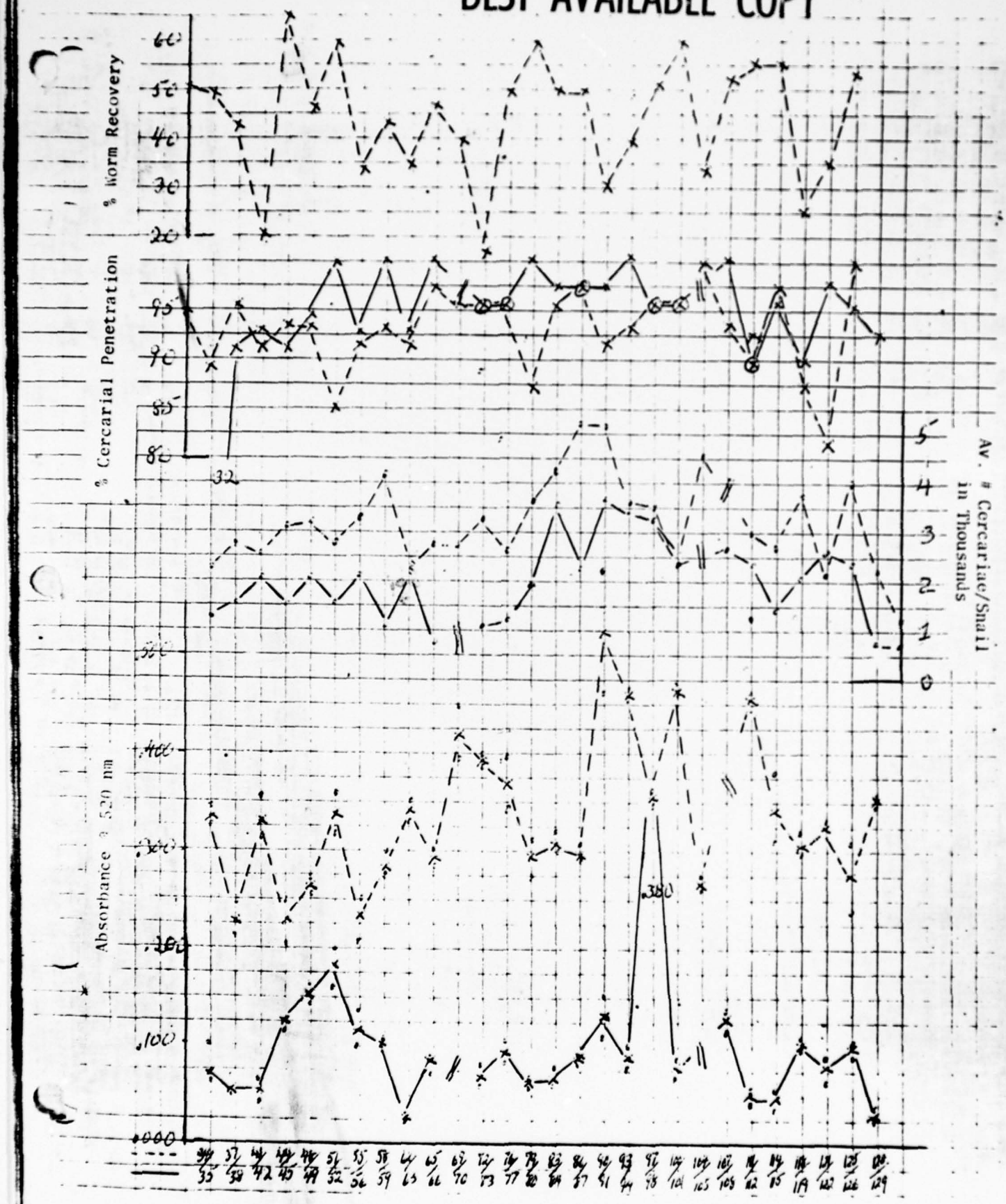


Figure 1

It appears that in these general terms, the curves of enzyme activity, cercarial production, and cercarial penetration all followed the same general pattern but there was no daily correlation. Maturation of adult worms did not conform to this pattern, such maturation probably being affected by numerous conditions within the mouse host which are poorly understood.

While the difference between 90 and 94% of cercariae penetrating may not seem impressive, distribution of the 81 samples of 50 cercariae per mouse during the three periods should be considered. Penetration of 45 or less of the 50 cercariae used to expose each mouse has in the past proven to indicate poor penetration and to result in low worm burdens. During the initial period, only 56% of the cercarial samples penetrated well on this basis; during the mid-period, 73%; and during the final period, only 50%.

Snails deaths did not appear to be directly related to the daily trends in enzyme activity, cercarial production or infectivity. Average numbers of deaths between collection days during the 3 periods were 13, 9 and 13 respectively.

Data from the replication of the first experiment are presented in Figure 1. While this was to be a replication, it was unavoidably not an exact one. After infection the snails became infested with rotifers. This is an adverse condition for production of large numbers of highly infective cercariae,

and is one of the snail maintenance conditions proposed in this contract for later testing for its effect on the parameters of concern. The rotifer infestation was controlled by scrubbing the snails with 10% ethanol followed by a water rinse.

By way of comparison of data from the original experiment and the replication, the following points are noteworthy. (1) The two curves of enzyme activity were not similar. The high enzyme activity during midpatency recorded in the original experiment was not duplicated in the replicate. Except for 2 brief periods of high activity, the curve of the replicate showed only the typical day-to-day variations without any sustained trends. Whether these differences were the result of the rotifer infestation remains to be shown in experiments in which the infestation is not controlled. It should be emphasized that only the patterns of enzyme activity are comparable here and not the levels, since the Azocoll substrates were not the same and the numbers of cercariae per point were different: 3500 in the broken line curve and 50 in the solid. The latter variations were necessary to provide absorbance readings which were within linearity of the spectrophotometer. (2) Curves presenting the average number of cercariae per snail on each cercarial collection day (cercariae/snail) for the 2 experiments did appear to be similar, although regression curves have not yet been plotted.

High cercarial production during snail midpatency was characteristic of both experiments. (3) Cercarial production expressed as cercariae/snail was consistently lower from the snails with rotifer infection than from those without rotifers. (4) There was no apparent correlation of cercarial infectivity for mouse hosts either between the 2 experiments or with the other parameters with each experiment. This was true for both penetration and worm burdens. It is of importance, however, here, to be aware of a technicality which was overlooked thus far and which will be considered in the future. Cercariae for application to mouse skin were not randomly pipetted into the mouse exposure vessels as should be done. It has been our custom for most purposes to select for penetration cercariae from concentrations at the surface of the cercarial suspension. This procedure automatically selects the most vigorous cercariae and so should not be used in these experiments. Here, a representative sample of the whole cercarial collection is required for testing infectivity.

B. Results of study of cercariae from snail infections with 1 miracidium are graphed in Figure 2. Unfortunately, the 5 snails isolated for cercarial collections did not produce enough cercariae for calculating the average cercarial emergence per snail or for data collection over a long period of time. This experiment is being repeated with a larger

Legend for Figure 2. Activity of enzyme secreted by cercariae from snails exposed to 1 miracidium each compared with the infectivity for mice of the cercariae from the same pool.

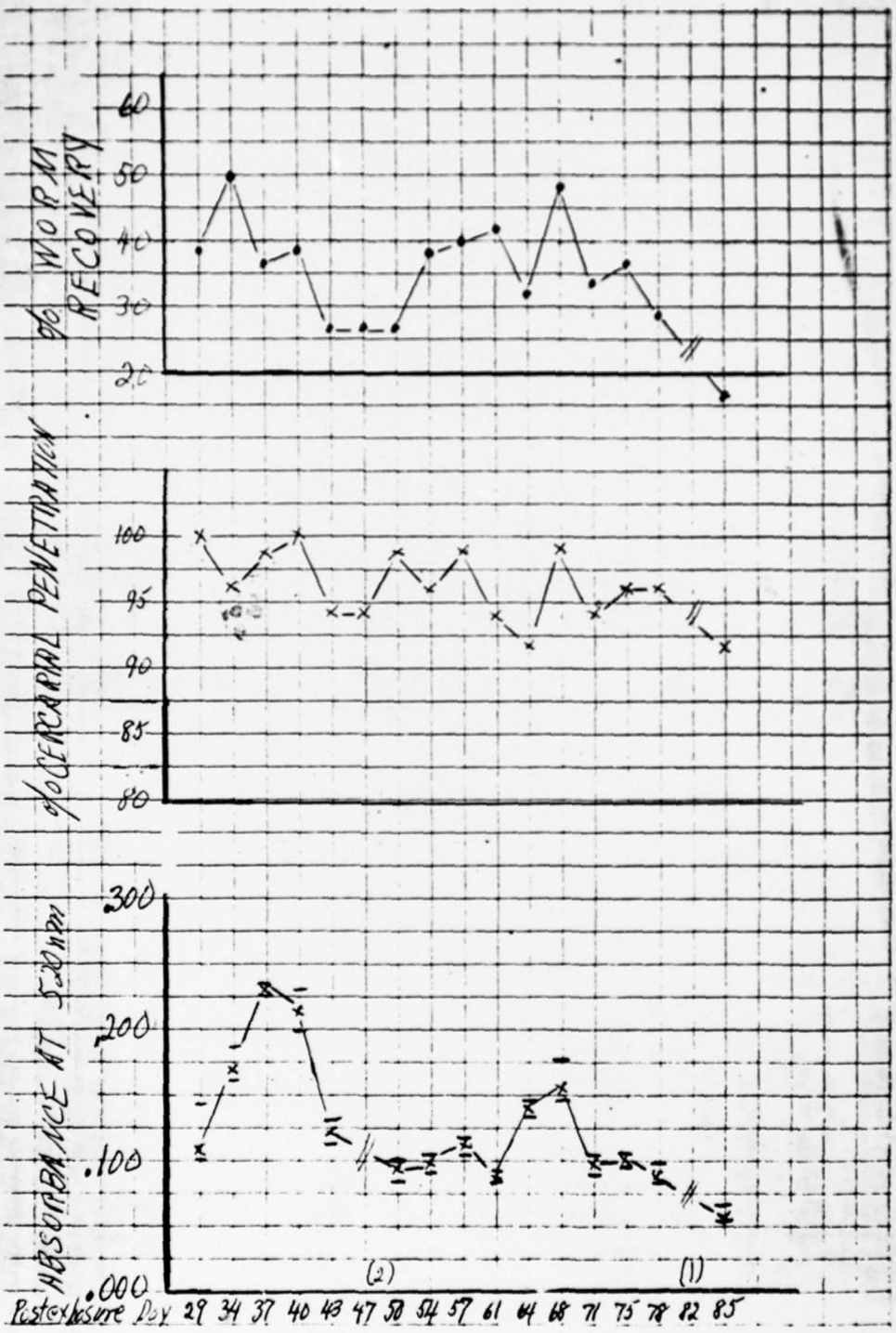


Fig. 2

number of snails. The only similar trends among the curves shown in Figure 2 occurred from the 68th through the 85th days postexposure of the snails. At this time, enzyme activity, cercarial penetration and worm maturation all decreased.

OBJECTIVE II. INHIBITORY QUALITY OF THE SECRETED PREACETABULAR GLAND SECRETION

Background. Some years ago we showed that an initial exposure to cercariae of Schistosoma mansoni gave mice partial, local protection against a subsequent challenge presented within 2 days. Worm burdens of the challenge cercariae were reduced by 67% with a 1 hour interexposure interval; 50%, with 1 day. After a 1 week interval, there was no protection. The protection was for the most part against cercarial penetration. With a 1 hour interexposure interval, penetration was reduced by 20%; 5 hours, 21%; 1 day, 17%; 2 days, 12%. While the percentage reduction here does not seem as great as in worm burdens, this level of reduction of penetration is compatable with that of the worm burdens. There was no reduction in penetration after 3 days or thereafter. It is possible that both initial entry into skin and migration therein were inhibited. No mechanisms of protection have been identified.

After it became possible to collect preacetabular gland

secretion in vitro, the hypothesis was considered that the presence in the penetrated skin area of preacetabular gland secretion from the initial cercariae might be the protective condition. The secretion was tested in vitro for its inhibitory effect on secretion of enzyme by added cercariae.

METHODOLOGY

Preacetabular gland secretion (Preparation A) was collected after stimulation of varying numbers of cercariae by linolenic acid in a temperature gradient as described in Annual Report #1 of July 1976, the cercariae filtered out, and the activity against Azocoll assayed. The numbers of cercariae used were 5000, 2000 and 500 in 10 ml of water. For the test preparation (B), 500 cercariae were stimulated as above in 10 ml of preparation A secretion and a similar number were stimulated in water as the control (Preparation C). Azocollytic activities were read spectrophotometrically as above. Without inhibitor, enzyme activity in the test preparation B less the activity of the original secretion (A) should be equal to that of the control (C).

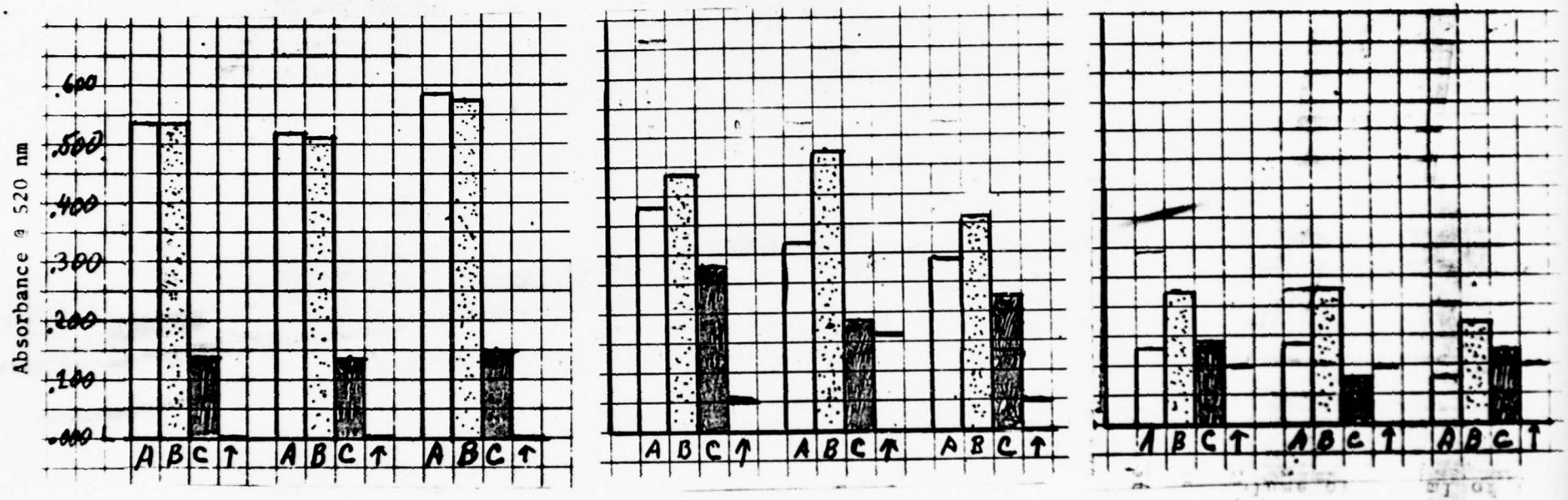
To establish where the inhibition occurred, that is, in the penetration response of the cercariae, in the emission of secretion from the glands, or in actual blocking of enzyme activity, cercariae were observed microscopically and their reactions compared in the stimulation vessels in previously

collected secretion and in water, and depletion of the preglandular contents in cercariae was assessed histochemically with purpurin indicator (Alizarin No. 6).

RESULTS

Harvested secretion from 5000 or more cercariae appeared to be completely inhibitory to the Azocollytic activity of enzyme from 500 added cercariae tested in it. Figure 3 records data from 3 replications, each showing averages of absorbance readings at 520 nm from triplicate enzyme-Azocoll tubes for each column. Harvested secretion from 2000 cercariae had less inhibitory effect, and from 500 cercariae, no effect. In the latter case, the difference between B and A was essentially equal to the control activity. (Fig. 3). These data constitute first tests. More sophisticated experiments must be done for confirmation.

As concerns the specific point of effectiveness of secretion inhibition, inhibition of cercarial response, emission of glandular contents or enzyme activity, observations made in triplicate with different secretion harvests showed that cercariae in secretion did respond to stimulation by attempting penetration of the lipidized surface as quickly and completely as did cercariae in water. Thus, secretion did not block the cercarial penetration response. Assessment of the degree of secretion histochemically in not complete.



A = 5000 cercaria secretion
B = 500 cercariae in A
C = 500 cercariae in water

A = 2000 cercaria secretion
B = 500 cercariae in A
C = 500 cercariae in water

A = 500 cercaria secretion
B = 500 cercariae in A
C = 500 cercariae in water

↑ = B-A

Figure 3. Enzyme activity against Azocoll as indicated.

OBJECTIVE III. CRYOPRESERVATION OF SCHISTOSOMULES FOR PREPARATION OF A VACCINE

METHODOLOGY

Various conditions for cryopreservation of schistosomules of S. mansoni have been tested by cooling the organisms, holding them in liquid nitrogen for 24 hr, thawing, and observing them for any obvious motion (with the dissecting microscope X 30 magnifications). The medium used was lactalbumin hydrolysate in Earle's salts and contained dimethyl sulfoxide (DMSO) as the cryoprotectant.

Conditions tested were: DMSO concentrations; cold equilibration time before cooling; type of cooling vessel; packed vs unpacked schistosomules; seeding vs non-seeding; seeding temperatures; cooling rate; and thawing rate.

RESULTS

In some experiments 50% or more of the organisms were obviously motile. Their infectivity for mice has not yet been assayed.

Conditions providing most organisms showing movement were: DMSO, 1 M; equilibration time of 7 minutes; roundbottomed tubes instead of tapered centrifuge tubes; nonpacked organisms; seeding at -6°C; and a slow cooling rate, less than 1° C/min. Data are not complete on the effect of different thawing rates.

Attempts to substitute methanol (17.5%) for DMSO have not been successful, although a preprint by Farrant and James does report partial success with this endeavor.

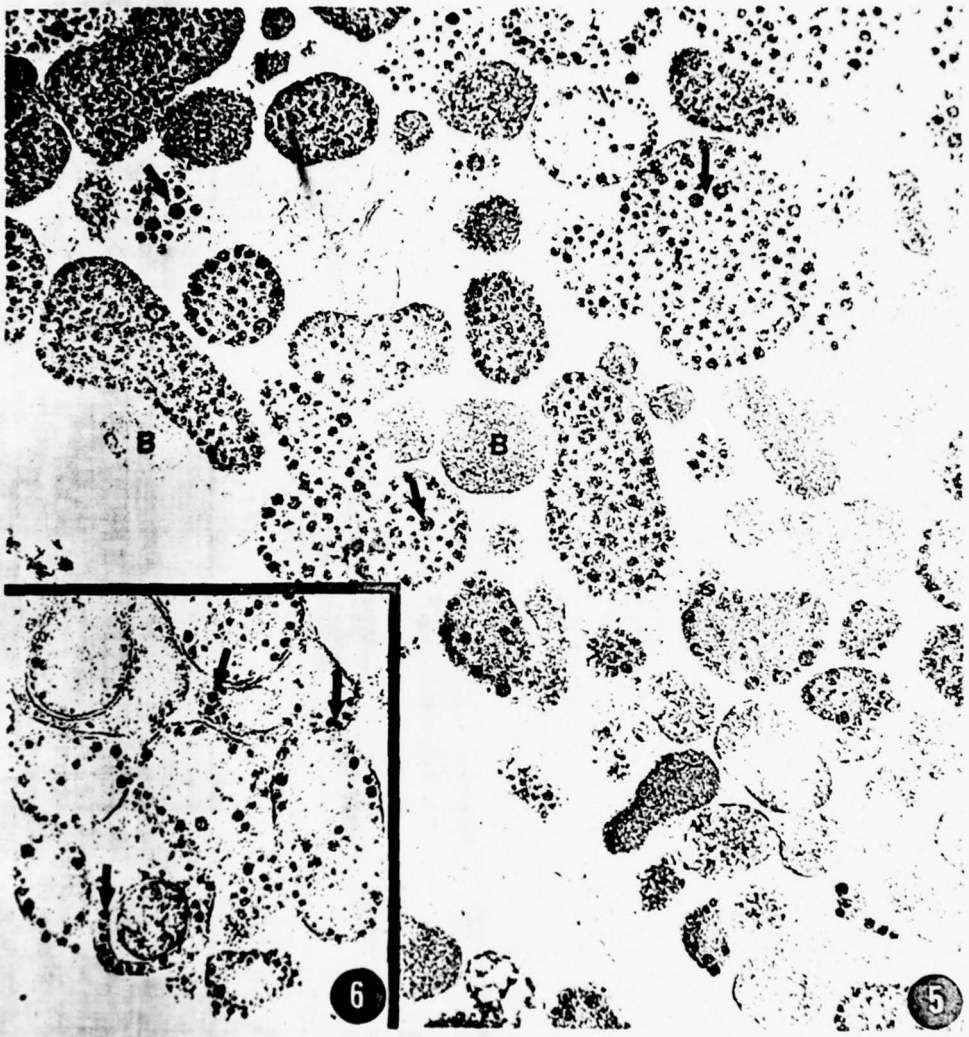
OBJECTIVE IV. UNDERSTANDING THE FINE STRUCTURAL MORPHOLOGY OF CERCARIAL PENETRATION ORGANS

METHODOLOGY

Calcium is known to be present in high molar concentration in the preacetabular glands of S. mansoni cercariae. In collaboration with Dr. Charles Dorsey at NMRI calcium was localized within the secretion granules in the glands. Cercariae were concentrated, and incubated in one of the cytochemical reagents useful in detection of calcium (potassium oxalate, potassium pyroantimonate, chloranilic acid or silver nitrate), and studied as appropriate in polarized light or fixed in gluteraldehyde and prepared for electron microscopy.

RESULTS

Calcium, apparently largely in the ionic state, was localized in cercariae in only one of the two types of secretion granules present in the preacetabular glands (Fig. 4) and in penetrated skin, namely, the type with electron lucid spots. The metallic cation was concentrated in the electron lucid areas. Absence of calcium in the homogeneous-appearing type of granule demonstrated that the two types of granules were dissimilar cytochemically as well as in ultrastructural appearance.



Electron micrograph of the fundus and duct (inset) of a cercarial preacetabular gland showing calcium localized in the electron-lucid areas of type A secretory granules. The electron-opaque precipitate is from the calcium pyroantimonate reaction. X 35000.

OBJECTIVE V. IMMUNOGENICITY OF COLLECTED PREACETABULAR GLAND SECRETION

METHODOLOGY

In collaboration with Drs. Patricia Minard and Darwin Murrell at NMRI, the immunogenicity of collected secretion was studied. Secretion was collected from cercariae stimulated by skin surface lipid. Protein concentrations were recorded; proteolytic activity was assayed against Azocoll; the secretion was fractionated by disc gel eletrophoresis, molecular sieve column chromatography and isoelectric focussing; and mice were immunized according to various schedules of injections of the secretion in adjuvants: Freund's complete or incomplete or alum. Sera were tested for antibody by passive cutaneous anaphylaxis assay, double gel diffusion and protection experiments.

RESULTS

Preacetabular gland secretion is a heterogeneous mixture of components containing both protein and carbohydrate. At least two of the components have proteolytic activity which may be important in the penetration process. With the protocols employed, immunization elicited IgE and/or IgG antibody response. Neither the cercarial secretion antigen alone nor in concert with adult freeze-thaw antigen was protective as shown by

absence of reduction in worm burdens of mice after immunization.

Failure to protect in any of these systems probably reflects a failure to induce all necessary types of responses which, acting in concert, afford protection. Immunization with cercarial secretion did induce IgE and/or IgG antibodies, but such were not sufficient in quality and/or quantity to protect mice.

OBJECTIVE VI. COMPARISON OF FINE STRUCTURE AND DEVELOPMENT OF CERCARIAE IN IN VITRO AND IN VIVO SCHISTOSOMULES

BACKGROUND

Perhaps the most critical aspect of the infective process, as seen by the parasitologist, is the postpenetration phase during which the schistosomes are in skin and thus easily available for experimentation. During penetration, the parasites transform quickly from free-living aerobic cercariae to post-penetration parasitic, anaerobic larvae called schistosomules. While the change occurs under natural conditions during entry into skin, the specific stimulus(i) is not known.

Schistosomules are needed in large numbers for immunological studies. Their harvesting after penetration of the skin in situ is timeconsuming. More important, their contact with host plasma makes them useless for some immunological studies. Therefore, several artificial means have been devised for the production

in vitro of schistosomules: mouse or dried rat skin penetration, shear pressure, centrifugation and temperature manipulation, treatment with linolenic acid, intraperitioneal maintenance.

A successful method for cultivating schistosomules has also been developed.

Many immunological studies are now being done with artificially-induced in vitro-produced schistosomules, but a comparative examination of their status as schistosomules and their histological development has not been made. There is, therefore, a need for an assessment of the status of schistosomules produced artificially in vitro. Using cercariae and true in vivo schistosomules for comparison, the histological and cytological changes which occur in schistosomules collected in vitro and developed in culture, together with the rate of development, is being studied. To date only the shear pressure artificial method has been used. Schistosomules produced by the other artificial techniques will also be investigated. The following questions are posed:

(1) How closely do artificially produced schistosomules conform to the definition of true schistosomules recovered from penetrated skin?

(2) What is the degree and rate of transformation in in vitro and in vivo schistosomules?

(3) What is the transforming stimulus(i)?

METHODOLOGY

In vitro and in vivo schistosomules were examined after 1, 3, 6, 24, 72, 96 and 120 hours. Schistosomules were harvested in vitro by subjecting cercariae to shearing forces created by 14 passages through a 21 gauge needle and cultured for the above times. In vivo schistosomules were obtained after cercariae placed on the ears of anesthetized mice had penetrated the skin. After the above time periods, the mice were sacrificed, the exposed areas of the ear excised and the dorsal and ventral skin layers separated and minced, freeing the organisms. Schistosomules were obtained from the lungs by mincing. They were subsequently incubated for the periods listed. They were obtained from both ear and lungs after 96 hours.

Cercariae in vivo and in vitro were compared with regard to the following differences. Cercariae are water-adapted; tailed; rigid and precise in silhouette; CHR positive (the cercarial glycocalyx (antigen) combines with an electrophoretically fast-moving gamma globulin (antibody) of immune serum to form an envelope); the acetabular glands are full of secretions as demonstrated histochemically with purpurin and PAS; there is no development in culture. In contrast, schistosomules are water-intolerant; tail-less; worm-like in appearance and locomotion; CHR negative; have emptied their acetabular glands; and develop in culture.

Cercariae and in vivo and in vitro schistosomules were prepared for electron microscopy for an extensive comparative study of their histological development.

RESULTS

At the optical microscope level, unstained schistosomules produced by passage through a 21 gauge needle, appeared similar in behavior and morphology to those produced in vivo. Both were tail-less and worm-like in appearance and locomotion.

Approximately 99% of all in vivo schistosomules were water intolerant, dying within 15 minutes, death being determined by the accepted criteria of death of schistosomules: loss of movement, loss of flame cell activity, extrusion of ventral sucker, bubbling of cytoplasm through membranes and loss of methylene blue exclusion capability. In vitro schistosomules became water intolerant more slowly, 95% in 48 hours (see table below).

On the other hand, in vitro schistosomules developed successfully in culture, about 70% living after 120 hours. This is about the same as in vivo schistosomules, roughly 35% of which die in skin. By contrast, cercariae did not survive in culture.

Results of the CHR test, the PAS postacetabular gland assessment and the EM data are not complete. All the organisms, that is, cercariae, and in vivo and in vitro schistosomules, have been fixed and embedded for sectioning and for histochemical analysis using PAS for all the time periods listed.

	TIME IN HOURS							
	1	3	6	24	48	72	96	120
WATER TOLERANT								
IN VIVO SCHISTOSOMULES	1%							
IN VITRO SCHISTOSOMULES	99%	95%	95%	67%	5%			
LIVING IN CULTURE								
IN VITRO SCHISTOSOMULES	95%	95%	90%	90%	76%	70%	70%	70%
CERCARIAE	99%	95%	92%	60%	30%	2%		
PREACETABULAR GLANDS EMPTY								
IN VIVO SCHISTOSOMULES	99%							
IN VITRO SCHISTOSOMULES	1%	5%	7%	13%	83%	98%		
CERCARIAE	0	0	0	dead				

DISCUSSION

Results of evaluating cercariae which were harvested regularly twice a week from 3 isolated groups of snails, and tested for emergence level, activity of secreted enzyme and infectivity made one point clear: variability was a prime characteristic of all the parameters. It was not only obvious in the day-to-day results, but

also in the pattern of longer trends, which were often of weeks' duration. It is apparent that the data recorded for the different parameters in the first experiment for cercariae from multiply exposed snails with the same patent period (the same date of exposure) did not show a daily correlation. The general overall patterns, on the other hand, were common for enzyme activity, cercarial emergence, and cercarial penetration: lower for about the first month and the final 2 weeks, and higher during the mid period. Neither snail deaths nor worm burdens followed this pattern.

In an intended replicate experiment, the general trends discussed above were not noted, nor was any correlation evident among the different parameters. The fact that the snails were infested with rotifers may explain the lack of correlation. One more replication, without rotifers, is needed to check for possible reproducibility of patterns. Such is in progress.

Data for cercariae from 1-miracidium snails with the same exposure date confirmed the high variability and the absence of any daily correlations among the parameters. They also affirmed a similar general overall pattern for enzyme activity and cercarial penetration. The curves present an overall general decrease. Worm burdens again did not follow the above pattern, although by all 3 criteria - enzyme activity, cercarial penetration and worm burdens - the pattern was strongly downward during the final 15 days. Unfortunately, cercarial harvests were not large enough to permit

calculation of the average daily numbers of cercariae emerging per snail. The patterns, however, were not similar to those of the first experiment with cercariae from a group of snails exposed to 8 to 10 miracidia.

It seems probable that worm burdens will not correlate very closely with any of the other parameters, possibly because of the tremendous influence on worm development of the internal host environment.

Judgment should be reserved on correlation between enzyme activity and infectivity in terms of cercarial penetration because the cercariae used for penetration were unwittingly selected for vigor during our process of concentrating them for counting.

Final analysis must await replications (in progress) of these experiments, after which the expected limits of variation can be set and base lines of enzyme activity and other parameters can be established for our conditions.

The second interest area was concerned with a possible inhibitory quality of concentrated collected secretion on further enzyme secretion into it by added cercariae as tested by activity levels against Azocoll. That an inhibitory quality of collected cercarial secretion may be demonstratable and may play a role in prevention of infection of mice <u>in vivo</u> is of great interest. There is a possibility that in this quality may lie the explanation of a local transient protection of mouse hosts against hyperinfection reported by Stirewalt in 1953. Should

such inhibitory quality be confirmed, the target activity must be identified. Observation of cercariae in collected secretion indicated that the cercarial penetration response was not blocked. It may be that added cercariae in concentrated collected secretion did not emit their preacetabular secretion as completely as did control cercariae in water, to the end that enzyme concentration was reduced. Or, of course, a true enzyme inhibitor might be present. This may be difficult to prove since activity of the test enzyme is superimposed on that of the original collected secretion.

These experiments seem worth continuing. If the presence of secretion from initial penetrants on and in skin can be shown to be the means of preventing subsequent parasite larvae from entering into and migrating in skin, this finding can have significance for our understanding: (1) of the biological relationships between hosts and parasites at this stage of infection; (2) of possible mechanisms of early host protection (although for a maximum of 2 days); (3) of a way by which parasites insure against host death by overwhelming infection; and (4) perhaps eventually of a design for a method of prophylaxis.

Our third objective is to develop a technique for storing schistosomules so as to retain their infectivity. The capability

of storing irradiated schistosomules is prerequisite to their use as a vaccine. The schistosomules must be infective. Results of our trials of cryopreservation of these organisms have made us hopeful of success, and defined some of the optimal conditions for preserving these organisms so they can be recovered living and motile. When optimal conditions have been defined, infectivity of the stored schistosomules for mice will be tested. This work is in collaboration with Dr. Darwin Murrell at NMRI, who has found that initial exposure of mice to irradiated living schistosomules protects against up to 90% of challenge cercariae.

Fourthly, description of the fine structure of the larval parasite organs used during skin penetration is an essential aspect of understanding the process of infection by schistosomes. Mucus from the postacetabular glands, and enzyme(s) and calcium from the preacetabulars are known to be 3 of the acetabular gland products. Surprisingly large concentrations of calcium were encountered by others in the preacetabular glands. Its function appears to be to regulate enzyme activity, protecting cercarial tissues from it by the high concentrations in the glands and stimulating it by low concentrations as it diffuses through skin. Our aim, to localize the calcium and to ascertain whether it was present not only in cercarial glands but also in penetrated skin, was achieved. It is our further aim to learn the fate of this cation. in skin as the granule membranes degenerate and release it.

Efforts in a fifth interest area were directed toward

ascertaining the immunogenicity of preacetabular gland secretion, since it is this gland product which is emitted into skin and which diffuses widely through epidermis and dermis around migratory parasites. Only the first steps toward this goal have been taken.

The fact that immunization with cercarial secretion produced IgE and/or IgG antibodies in mice but did not protect them against challenge cercarial exposures, means that some conditions required for protection were not fulfilled. Protection may require other antigen concentrations, concurrent and/or serial presentation of other antigens, or other immunization schedules. In addition to the humoral, the cellular response to injected cercarial secretion should be studied. This is so planned.

Finally, we have taken advantage of the fact that an electron microscopically trained parasitologist, Dr. Carolyn Cousin, is spending a sabbatical year in this laboratory on an NIH-MARC Fellowship. With her help, we have begun a study of the validity of in vitro artificially-produced schistosomules as compared with in vivo true (skin penetrated) schistosomules. The comparison includes behavioral assessment, fine structural changes and rates of development.

It appears at present that the best artificially-produced schistosomules are provided by the shear stress method. Comparison of these with true schistosomules revealed that the organisms do indeed conform to the presently used criteria for this stage

of parasite (See Results) but transform from the cercarial stage much more slowly than do true ones. Electron microscopic examination is in progress.

This study is of importance because, even though much use is being made of the artificially-produced schistosomules, their validity as schistosomules has not been proven. It is our purpose to test the validity of all types of artificially-produced schistosomules.

CONCLUSIONS

1. Expected limits of variability for enzyme activity in collected preacetabular gland secretion of cercariae of Schistosoma mansoni have been defined for our conditions.

2. Day-to-day variations in enzyme activity did not appear to be related to other parameters, but long-term trends may be: to cercarial emergence levels and possibly to cercarial penetration capability. Reproducibility of the curves is under test.

3. Curves representing enzyme activity, cercarial penetration and worm burdens were dissimilar when cercariae were from snails with and without rotifer infestations and from snails with infections developing from single and multiple miracidial exposures.

4. An enzyme activity-inhibiting quality was suggested in concentrated collected secretion, but whether its point of effectiveness was anti-enzyme or anti-secretion has not been established.

5. Collected secretion was immunogenic in that it raised the immunoglobulines IgE and/or IgG in mice. With the immunizing schedules used, secretion was not protective.

6. Methodology is available for cryopreservation of schistosomules which are living and motile upon thawing after storage at -196°C. The techniques need improvement to insure recovery in quantity of schistosomules which retain not only motility but, more importantly, infectivity.

7. Calcium in preacetabular glands, a bivalent cation which appears in vitro to have a regulatory function for the penetration enzyme, was shown to be concentrated in the electron-lucid areas of one of the two types of preacetabular secretion granules. It was present in penetrated skin as well as in the cercarial glands, and was largely in the ionic state. The two types of granules were thus chemically different.

8. Organisms stimulated to transform from cercariae to schistosomules by the shear stress method, did so transform. The change was much slower for artificially-produced than for true schistosomules, requiring up to 48 hours for the former as compared with 1 hour or less for the latter.

SIGNIFICANCE

A more comprehensive integrated picture is needed of the invasive process during which schistosome cercariae penetrate vertebrate host skin and migrate in it, concurrently transforming from cercariae to schistosomules. Such picture is

pertinent to immunological, biological, biochemical, prophylactic and chemotherapeutic investigations. It is our purpose to contribute as completely as possible to this total picture.

Our investigations of the expected normal level of secreted cercarial enzyme activity contribute to understanding the migratory phase of the invasive process, since this enzyme(s) is secreted into skin by penetrants and apparently acts on some element of the host tissue thus aiding migration through it. Establishment of normal enzyme activity levels is prerequisite to reasonable attempts to change this activity by reducing it.

Reproducibility of base line results showing the variations and patterns of cercarial enzyme activity, cercarial emergence levels, and cercarial infectivity in terms of penetration and mature worm burdens is being investigated. Should it be established, the function of the penetration enzyme would be clarified.

An obvious corollary to the enzyme activity studies is elucidation of the role of calcium in enzyme control. Localization of the calcium is related to this aspect of the work.

Also closely related to the study of secreted enzyme activity levels is the attempt to find means of inhibiting this activity, or at least reducing in some way the cercarial capability of penetrating skin. Should an inhibitory function of concentrated

collected secretion be confirmed, this would be of major value in the long-term approach to such an objective. Further study along this line should contribute to elucidation of the inter-related biology of the hosts and parasites, mechanisms of early host protection, parasite demography, and prophylaxis.

Related to the prophylactic aspects of control of schistosomiasis, are the investigations of immunogenicity of cercarial secretions and those on cryopreservation of irradiated schistosomules. Both may provide information contributory to the design of penetration inhibition or a vaccine.

Validation of the schistosomular status of artificially transformation-stimulated cercariae and delineation of their development are in the area of vaccine production also, since many of these organisms are being used in immunological experiments. Understanding the comparative rates of their development, not only in culture but also in ear and lung tissue in vivo, will give information necessary for planning attacks on the parasites in these host tissues before maturation of the worms.

CURRENT REPORTS AND PUBLICATIONS

(a) Stirewalt, M.A. in press. Quantitative collection and proteolytic activity of preacetabular gland enzyme(s) of cercariae of Schistosoma mansoni. American Journal of Tropical Medicine and Hygiene.

(b) Dorsey, C.H. and Stirewalt, M.A. in press. Schistosoma mansoni: localization of calcium-detecting reagents in electron-lucid areas of specific preacetabular gland granules. Zeitschrift fur Parasitenkunde.

(c) Minard, P., Murrell, D. and Stirewalt, M.A. 1977. Proteolytic, antigenic and immunogenic properties of Schistosoma mansoni cercarial secretion material. American Journal of Tropical Medicine and Hygiene, 26, 491-499.

(d) Stirewalt, M.A. 1976. Activity of enzymes secreted by cercariae of Schistosoma mansoni. Program and Abstracts of the 51st Meeting of the American Society of Parasitologists (Abstract).

(e) Dean, D.A., Minard, P., Stirewalt, M.A., Murrell, K.D. and Vannier, W.E. 1977. Role of Schistosoma mansoni eggs in resistance of mice to reinfection. Proceedings of the 61st annual meeting of the Federation of American Societies for Experimental Biology, April 1977, Chicago, Ill. and the 52nd annual meeting of the American Society of Parasitologists, Las Vegas, Nevada, August 1977.

(f) Dean, D.A., Stirewalt, M.A., Murrell, K.D. and Vannier, W.E. 1977. Mechanisms involved in immune destruction of schistosomes. 52nd Annual Meeting, American Society of Parasitologists, Las Vegas, Nevada, 14-19 August, 1977. (Abstract).